Pathogenicity and environmental tolerance of commercial and UK native entomopathogenic nematodes (*Steinernema* and *Heterorhabditis* spp.) to the larvae of mosquitoes (*Aedes aegypti* and *Ochlerotatus detritus*)

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Abstract

Many vector mosquito species have evolved resistance to chemical insecticides and the search for novel biological control strategies warrants further attention. Entomopathogenic nematodes (EPNs) (*Heterorhabditis* and *Steinernema* spp.) have been developed as biological control agents for use against agricultural pests but whether they could be used to control aquatic mosquito larvae warrants further research. We exposed *Aedes aegypti* and *Ochlerotatus detritus* larvae to commercially available (*Steinernema feltiae, S. carpocapsae*, *S. kraussei* and *Heterorhabditis bacteriophora*) and wild isolated (*S. affine* and *S. glaseri*) EPNs and monitored survival over 7 days. We also exposed EPNs to water with a range of salinities and pHs found in the marshland habitats of British mosquitoes. *Ae. aegypti* and *O. detritus* were killed by commercial EPNs, but wild strains were unable to kill *Ae. aegypti* yet did kill *O. detritus.* All EPNs were capable of tolerating a wide range of pHs but showed variable tolerance to different salinities. EPNs could be used as an alternative to chemical insecticides but target species and habitat may influence choice of EPN strain in control operations.

Introduction

Mosquitoes from the genus *Aedes*, *Culex* and *Anopheles* are some of the world's biggest killers as they vector infectious arboviruses (e.g. dengue and Zika) and the malarial parasite *Plasmodium* (Naghavi et al. 2015). These pathogens cause nearly 350 million cases and nearly half a million deaths per year (Moyes et al. 2017). Due to the rise in insecticide resistance in many mosquito vector species (Moyes et al. 2017; Ranson 2016) alternative control strategies are needed urgently (Achee et al. 2019; Thomas, 2018). Non-chemical approaches include the use of *Bacillus thuringiensis israelensis* (*Bti*) (Lacey 2007; Zhang et al. 2017), the sterile insect technique (Lees et al. 2015) and the introduction of *Wolbachia* infection into mosquito populations (O'Neill 2018; Gomes and Barillas-Mury 2018). The use of parasitic nematodes, particularly entomopathogenic nematodes (EPNs), warrants further investigation. For example, mermithid nematodes such as *Romanomermis culicivorax* and *Romanomermis iyengari* (Koylinski et al. 2012) are natural parasites of mosquitoes and will successfully infect and kill mosquitoes. However, they are difficult to mass-produce as they must be grown *in vivo* in mosquitoes limiting their practicable use. An alternative is the use of entomopathogenic nematodes from the families Steinernematidae and Heterorhabditidae, which are natural parasites of many insects and have been developed as biological control agents used widely in agriculture (Campos-Herrera 2015). Nematodes are applied to soil where they seek out insect hosts and are attracted to host specific cues and carbon dioxide (Dillman et al. 2012). They penetrate inside and release a symbiotic bacterium (*Xenorhabdus* spp. for *Steinernema* and *Photorhabdus* spp. for *Heterorhabditis*) (Ciche and Ensign 2003; Martens et al. 2003), which proliferates and produces an abundance of toxins, invasins and extracellular enzymes that can kill the host in 24-48 hours (Bisch et al. 2016). The nematodes then feed on the decaying cadaver, reproduce, and when this food source is exhausted, the offspring turn to dauer stage nematodes and search for more hosts to parasitise.

EPNs have been successfully shown to control a range of terrestrial insect pests (Campos-Herrera 2015), however there have been mixed results investigating whether *Heterorhabditis* and *Steinernema* could be used to kill the aquatic stages of mosquito vectors. *Steinernema carpocapsae* is able to penetrate through the gut wall of *Aedes aegypti*, *Aedes stimulans* and *Aedes trichurus* resulting in host death (although many are encapsulated) (Welch and Bronskill 1962; Bronskill 1962). Whilst Dadd (1971) recorded *Culex pipiens* ingesting *S. carpocapsae*, often hundreds at a time, they would fail to make it into the haemocoel and not cause mortality. Similarly, Poinar and Kaul (1982) showed that *C. pipiens* would ingest *Heterorhabditis bacteriophora*, but at high doses they would escape melanisation and cause death. Recently, de Oliveira Cardoso et al. (2015) found that two *Heterorhabditis* species (two strains of *Heterorhabditis indica* and *Heterorhabditis baujardi*) could kill *A. aegypti* but *S. carpocapsae* could not. Peschiutta et al. (2014) reported *H. bacteriophora* could cause 84% mortality in *Ae. aegypti* and Cagnolo and Almirón (2010) showed *Steinernema rarum* could kill *Culex apicinus*. Dilipkumar et al. (2019) demonstrated that *Steinernema abassi* exerted high mortality against *A. aegypti*, *H. indica* against *A. stephensi* and *S. siamkayai* against *C. quinquefasciatus*. Although not a mosquito, Edmunds et al. (2017) also showed that aquatic stages of the non-biting midge *Chironomus plumosus* were rapidly killed by *Steinernema feltiae, S. carpocapsae, Steinernema kraussei* and *H. bacteriophora*. Yooyangket et al. (2018) also showed that as well as nematodes, the bacteria isolated from native EPNs (*Xenorhabdus stockiae* and *Photorhabdus luminescens* subsp. *akhurstii*) were highly toxic to mosquitoes (*Ae. aegypti* and *Aedes albopictus*). With these conflicting reports on the susceptibility of mosquitoes and varying degrees of nematode pathogencity we decided to investigate whether commercially produced and field isolated EPNs could be used to kill the larval stage of two species of mosquito under laboratory conditions. We concentrated on *Ae. aegypti* and *Ochlerotatus detritus*, a medium sized

mosquito typically found in coastal areas throughout the U.K. (Clarkson and Setzkorn 2011; Blagrove et al. 2016). It is highly halo-tolerant and gravid females oviposit in salt-marsh habitats where they lay their eggs on the soil of shallow pools, which are subject to regular tidal inundations (Service 1968; Becker et al. 2010). It is multivoltine and an opportunistic biter of both humans and animals, and is regularly noted as one of the top three recorded nuisance-biting mosquitoes in the U.K. (Medlock et al. 2012). *O. detritus* has been found to be able to transmit Japanese encephalitis and West Nile virus under laboratory conditions (Mackenzie-Impoinvil *et al.*, 2015; Blagrove *et al.*, 2016). Owing to the sensitive nature of the habitat in which *O. detritus* is found, it can be difficult to control using standard chemical controls (Brown et al., 2019). Larger scale strategies are often co-ordinated by local governing authorities such as digging deeper channels in marshy or boggy areas where specific species such as *Ochlerotatus* spp. are known to breed (James-Pirri et al., 2009; Rey et al., 2012) or using Bti with only limited success (Clarkson and Setzkorn, 2011) Therefore, new control strategies for this species need to be examined and the susceptibility of *O. detritus* to EPNs has never been tested. As mosquitoes such as *O. detritus* live in saltmarshes that are subject to extreme and variable environmental conditions, we investigated whether EPNs could survive under a range of pHs and salinities.

Our aims were: 1. To discover whether EPNs could cause mortality to *Ae. aegypti* and *O. detritus* 2. To examine whether commercially available or wild isolated EPNs were more pathogenic 3. To investigate whether there was a difference in susceptibility of the two mosquito species exposed to EPNs 4. To understand the survival of EPNs in a range of pHs and salinities commonly found in the environment of *O. detritus*. Demonstration of pathogenicity of EPNs could lead to novel and effective method of mosquito control.

Materials and Methods

Insect sourcing and rearing

Aedes aegypti (New Orleans strain – Stell et al. 2012) eggs were obtained from the Liverpool Insect Testing Establishment (LITE) at the Liverpool School of Tropical Medicine. Egg papers were floated in a flat-bottomed tray containing approximately 7 cm depth of nutrient-rich medium (cat biscuits in distilled water allowed to stagnate for a minimum of 24 hrs). Hatched larvae were kept at room temperature (19-25°C) until they reached third instar. *Ochlerotatus detritus* larvae were collected from a pool with salinity of 25 ppt at Little Neston (N 53° 16' 40.771" W 3° 4' 6.967"). Once transported back to the laboratory at Liverpool John Moores University (LJMU) they were incubated at 15°C in containers of brackish water collected with the larvae and allowed to develop to third instar. The temperature of 15°C was chosen as temperatures above this adversely affect the survival of *O. detritus* and this is a representative temperature of salt marsh pools (Currie-Jordan, 2019).

Entomopathogenic nematode strains

Commercial EPN strains (*S. feltiae, S. carpocapsae*, *H. bacteriophora* and *S. kraussei*) were supplied as partially desiccated nematodes by BASF Agricultural Specialities, U.K at ~6 million nematodes per pack. From a recent survey of EPNs from around the U.K. (Edmunds et al. 2018) several species and strains of *Steinernema* were isolated and cultured for use in this study including *Steinernema affine* (strain 173) and *Steinernema glaseri* (strains 93, 119 and 367). Briefly, Edmunds et al. (2018) baited 518 soil samples from around the U.K. with the waxmoth (*Galleria mellonella*) (a highly susceptible host). After 7 days any potentially EPN parasitised *G. mellonella* were placed on individual White traps (White, 1927) and nematodes were identified using molecular verification of the 18SrRNA gene. Using this approach 18 wild isolates of EPNs were isolated and cultured at LJMU and several strains were used in this experiment. In order to obtain sufficient EPNs for pathogenicity

assays the nematodes were sub-cultured using *G. mellonella*. Briefly, 1 ml of approximately 1,000 dauer stage *S. affine* 173, *S. glaseri* 93, 119 or 367 were pipetted onto a pre-moistened 10 cm Whatman filter paper and placed in a Petri dish. Ten *G. mellonella* larvae were added and the Petri dish sealed and stored at 20°C. Every 48 hours *G. mellonella* were examined for mortality and any dead were placed in a modified White trap (White 1927) and new dauer stage nematodes were collected after 14 days.

Survival of *Ae. aegypti* and *O. detritus* exposed to EPNs

As per standard mosquito testing procedures (WHO, 2005) 100 ml of distilled water and 0.025 g of crushed cat biscuit was added to 250 ml plastic cups (70 mm diameter top x 44 mm base x 80 mm height) with twenty-five L3 stage *Ae. aegypti* added to each cup. Both commercially available *S. feltiae, S. carpocapsae, H. bacteriophora* and *S. kraussei* and naturally isolated *S. affine* 173 and *S. glaseri* 93 and 367 were added at doses of 0, 2000, 4000 and 8000 to three replicate cups. The nematodes were quantified per ml using a stereomicroscope and then added directly to the water before addition of the mosquitos. To assess the survival of *O. detritus* exposed to EPNs, modifications were made to the assay since *O. detritus* is larger than *A. aegypti* and inhabits brackish water with a salinity of 25 ppt, therefore, in assays using *O. detritus* only 15 larvae were added to 100 ml of 25 ppt salinity water. Fifteen larvae were chosen as any more would affect their survival due to crowding (Edmunds, personal observation). After the nematodes were added, cups containing *Ae. aegypti* were incubated at 20°C and *O. detritus* assays were incubated at 15°C. Survival of the L3 larvae was monitored every 24 hours for 7 days. The numbers of dead, alive, pupated or eclosed individuals was recorded. Three cups were used for each treatment and the experiment was repeated three times.

Survival of EPNs exposed to different water salinities and pHs

Using a curved bottomed 96-well plate, 50 µl of saline solution (0, 10, 20, 30, 40, 50 or 60 ppt) was added to 12 wells in a 96 well plate. To separate wells, 1 EPN of each strain was added. The plate lid was sealed with Parafilm® and plates incubated at 15°C. The following EPNs were used: commercially available *S. feltiae*, *S. carpocapsae* and *H. bacteriophora* (no *S. kraussei* were used) and naturally isolated *S. affine* 173 and *S. glaseri* 93, 119 and 367. Survival was monitored every 24 h for 7 days. The EPNs were recorded as alive if they responded to prodding with a wire pick. The experiment was repeated three times. To examine the survival of EPNs exposed to different pHs a similar set up was used. Fifty microliters of water adjusted to pH 3, 4, 5, 6, 7, 8, 9 or 10 was added to 8 wells in a 96 well plate. Ten EPNs were added to each well, sealed with Parafilm® and incubated at 15°C. Nematode survival of *S. feltiae, S. carpocapsae, S. kraussei* and *H. bacteriophora* and naturally isolated *S. affine* 173 and *S. glaseri* 119 and 367 was recorded every day for 7 days and the experiment was repeated three times.

Data analysis

Survival of mosquitoes exposed to different doses and nematodes exposed to different pHs and salinities was analysed using Log-Ranked tests using OASIS (Yang et al. 2011).

Results

Survival of *Ae. aegypti* and *O. detritus* exposed to commercial and naturally isolated EPNs

There was a highly significant difference in the survival of *Ae. aegypti* larvae when exposed to all doses (2000, 4000 and 8000 nematodes) of commercially produced *S. feltiae*, *S. carpocapsae*, *S. kraussei* and *H. bacteriophora* compared to the control (0 nematodes) ($p \leq 0.001$) (Fig. 1A-D) with 70-80% of mosquito larvae dead within 6-7 days. In contrast, naturally isolated *S. glaseri* 93 had no effect on the survival of *Ae. aegypti* at doses of 2000 (*p*

= 0.098), 4000 (p = 0.1519) or 8000 EPNs (p = 0.1134) compared to the control (Fig. 1E). Similarly, the survival of *Ae. aegypti* was not affected by exposure to *S. glaseri* 367 or *S. affine* 173 applied at 2000, 4000 or 8000 EPNs compared to the control ($p > 0.05$) (Fig. 1F,G).

Commercial *S. feltiae*, *S. kraussei*, *S. carpocapsae* and *H. bacteriophora* caused significant mortality to *O. detritus* compared to the untreated control when applied at 2000 ($p \leq 0.001$), 4000 ($p \leq 0.001$) and 8000 nematodes ($p \leq 0.001$) (Fig. 2A-D) with rapid mortality observed e.g. for exposure to *S. feltiae* there was 90-100% mortality within 3 days. Similar to the commercial EPNs, naturally isolated *S. glaseri* 93 and 367 and *S. affine* 173 caused a significant difference in survival of *O. detritus* larvae when exposed to 2000 ($p \leq 0.001$), 4000 ($p \leq 0.001$) and 8000 nematodes ($p \leq 0.001$) compared to the untreated controls (Fig. 2E-G). Over the course of the experiment dead *A. aegypti* (and *O. detritus*) were examined for presence of EPNs that had penetrated into the larvae and many dauer juveniles were observed (Fig. 3).

Survival of EPNs exposed to different salinities and pHs

Commercial EPNs (*S. feltiae, S. carpocapsae* and *H. bacteriophora*) and naturally isolated *S. affine* 173 and *S. glaseri* 93, 119 and 367 differed in their tolerance to salinities of 0, 10, 20, 30, 40, 50 or 60 ppt over 7 days (Table 1). Survival of the commercial strain of *S. feltiae* and the wild isolate of *S. affine* 173 in 0 ppt was significantly greater than those exposed to 30, 40, 50 and 60 ppt ($p < 0.05$). This was similar to the survival of *H. bacteriophora* which was killed by salinities of 20, 30, 40, 50 and 60 ppt ($p < 0.05$). However, the other nematodes showed a mixed survival response when exposed to different salinities. For example, the survival of *S. glaseri* 367 was significantly affected by the extreme salinities of 10 and 60 ppt ($p < 0.05$). Whereas the survival of *S. glaseri* 93 was

significantly reduced by all salinities apart from 30 ppt ($p < 0.05$). Similarly, commercial *S. carpocapsae* was killed by all salinities apart from 50 ppt.

There was no significant difference in the survival of the commercial EPNs (*S. feltiae, S. carpocapsae, H. bacteriophora* and *S. kraussei*) and naturally isolated *S. affine* 173 and *S. glaseri* 119 and 367 exposed to pH 4, 5, 6, 7, 8, 9 and 10 over 7 days (Table 2).

Discussion

Previous studies examining the effect EPNs have on mosquitoes have reported mixed results suggesting that both EPN species and mosquito target have an effect upon pathogenicity. The results from our study show that exposure to commercial EPNs or strains isolated from the wild can have dramatic differences in the survival of *Ae. aegypti* and *O. detritus*. We found that field-collected EPN strains were avirulent towards *Ae. aegypti* but highly pathogenic to *O. detritus*. In general, the pathogenicity of field collected EPNs is compared to commercial preparations infrequently. Those few studies that do exist report conflicting results. McGraw and Koppenhöfer (2008) found that naturally isolated *S. feltiae* and *S. carpocapsae* were no less effective than their counterpart commercial strains against the annual bluegrass weevil (*Listronotus maculicollis*). Noujeim et al. (2015) observed higher mortality of sawflies (*Cephalcia tannourinensis*) when exposed to a natural strain of *H. bacteriophora* compared to a commercial strain. Bélair et al. (2013) compared the pathogenicity of commercial preparations of *S. carpocapsae* and *S. feltiae* with field-isolated strains towards black cutworm (*Agrotis ipsilon*) finding no difference between the virulence of the commercial and five strains of *S. feltiae*. However, they did find a more virulent strain of *S. carpocapsae* and four strains that were significantly less virulent than the commercial *S. carpocapsae*. Ultimately, natural variation in virulence plays a large role in the success of

these nematodes and should be considered when selecting the most suitable strain or species to combat specific pests in biological control programmes.

We found that our wild EPN isolates were highly pathogenic to *O. detritus*. The *O. detritus* larvae that were used in these experiments were collected from a naturally occurring population from Little Neston saltmarsh. These mosquitoes have benefited from the stringent legal protections on the area and only sporadic mosquito control measures such as *Bti* spraying for many years (Clarkson and Setzkorn 2011; Brown et al. 2019). Generally, natural infection of nematodes in British mosquitoes is low (Medlock and Snow 2008). Service (1977) reported finding larvae of *Ochlerotatus cantans* infected with several nematodes, but these were unidentified mermithids and not EPNs. To investigate this further we used *G. mellonella* baiting of several samples from the Little Neston area collected in a previous study (Edmunds et al. 2018) but found no EPNs, likely due to unsuitability of the heavy clay substrate of this habitat for EPNs (Kung et al. 1990). Therefore, it seems *O. detritus* may not come into contact with EPNs frequently, and perhaps therefore has not evolved defences against them, making them particularly susceptible to attack. However, it seems curious as to why the wild isolates were unable to kill *Ae. aegypti*. The *Ae. aegypti* New Orleans strain has been laboratory reared for many years (Stell et al. 2012), and since genetic diversity in long-established mosquito colonies is typically reduced (Lainhart et al. 2015, Azrag et al. 2016), it would be thought that lower genetic diversity may increase susceptibility, however, we found the opposite. The reasons for this resistance remain elusive. Perhaps these mosquitoes are more efficient at recognising and encapsulating nematodes, as observed in *Ae. aegypti* and *C. pipiens* (Welch and Bronskill 1962; Bronskill 1962; Poinar and Kaul 1982). Although it should be noted that this reponse seems to be specific to the field-collected strains as commerical preparations were able to kill *Ae. aegypti*.

Mosquito larvae are tolerant of a broad range of acidic and basic aquatic conditions (Clark et al. 2004). For example, *O. detritus* are found in salt-marshes with brackish water (measured at 25 ppt at Little Neston). Therefore, a range of salinities and pHs were tested to ascertain whether the EPNs were capable of tolerating mosquito habitats. The EPNs used in this study showed a mixed ability to tolerate a wide range of salinities. For example, commercial *S. feltiae*, *S. carpocapsae*, *H. bacteriophora* and field collected *S. affine* 173 were killed at salt concentrations of over 30 ppt but *S. glaseri* 367 was only killed at the lowest and highest concentrations (10 and 60 ppt). EPNs have previously been shown to cope well with high saline environments. For example, Griffin et al. (1994) showed that six strains of *H. bacteriophora* could survive for over 19 weeks in seawater. Thurston et al. (1994) even found that *H. bacteriophora* virulence was enhanced by low levels of salinity but higher concentrations affected survival. Our most salinity-tolerant strain was *S. glaseri* 367, which was the only EPN isolate from an extensive survey of Lundy Island (Edmunds et al. 2018). This extreme survival of saline conditions could potentially be due to adaption to the coastal conditions that prevail across such a small island. As well as salinity, we also investigated the tolerance of the EPNs to different pHs. We showed that commercial and UK native nematodes were remarkably resistant to water of a range of acid and alkali pHs. Studies on the effect of soil pH on EPNs have shown that they can survive and parasitise within a broad pH range but high alkaline content can act as a nematocide (Kung et al. 1990) and low pH significantly restricted infection by *S. kraussei, S. glaseri, S. scarabaei, H. bacteriophora* and *H. zealandica* (Barbercheck 1992; Koppenhöfer and Fuzy 2006). We have previously shown that EPNs sink to the bottom of water columns but remain alive and pathogenic (Edmunds et al. 2017). Mosquito genera exhibit different feeding strategies with *Anopheles* and *Culex* typically surface feeding, whilst *Aedes* feed on substrates and container walls (Yee et al. 2008; Skiff and Yee 2014) thus EPNs may be better suited for control of *Aedes*.

Ultimately, this research has shown that commercial preparations of EPNs are pathogenic to *Ae. aegypti* and *O. detritus* and that field-collected EPN species have reduced virulence to *Ae. aegypti*. These results mean there is potential for the production of a successful biological control agent for pestiferous mosquitoes (providing the results could be repeated in the field) but careful consideration should be given to whether endemic or commercial varieties should be used as well as EPN tolerance to specific habitat conditions.

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Figure legends

Figure 1: Survival of *Ae. aegypti* exposed to commercial EPNs: *S. feltiae* (A), *S. carpocapsae* (B), *S. kraussei* (C) and *H. bacteriophora* (D) and naturally isolated *S. glaseri* 93 (E) and 367 (F) and *S. affine* 173 (G) exposed to 0 (blue), 2000 (red), 4000 (green) and 8000 (purple) nematodes for 7 days. Bars represent ± one standard error.

Figure 2: Survival of *O. detritus* exposed to commercial EPNs: *S. feltiae* (A), *S. carpocapsae* (B), *S. kraussei* (C) and *H. bacteriophora* (D) and naturally isolated *S. glaseri* 93 (E) and 367 (F) and *S. affine* 173 (G) exposed to 0 (blue), 2000 (orange), 4000 (grey) and 8000 (yellow) nematodes for 7 days. Bars represent ± one standard error.

Figure 3: After 48 hours of infection *S. kraussei* had penetrated into *Ae. aegypti* larvae and were visible in the head of the larvae. Arrows point to dauer stage nematodes. Scale bar represents 100 μm.

Table 1: Mean percentage survival of commercial EPNs (*S. feltiae*, *S. carpocapsae* and *H. bacteriophora*) and naturally isolated *S. affine* 173 and *S. glaseri* 93, 119 and 367 exposed to 0, 10, 20, 30, 40, 50 or 60 ppt salinity on day 7 and *p* values (bold) from log rank statistical analysis when compared to the survival of nematodes exposed to 0 ppt salinity. *P* values < 0.05 are denoted with *

Table 2: Mean percentage survival of commercial EPNs (*S. feltiae*, *S. carpocapsae*, *S. kraussei* and *H. bacteriophora*) and naturally isolated *S. affine* 173 and *S. glaseri* 119 and 367

exposed to pH 4, 5, 6, 7, 8, 9 and 10 on day 7 and *p* values (bold) from log rank statistical analysis when compared to the survival of nematodes exposed to pH 7.